FORM PTO-1390 (Modified) (REV 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
29288.5600	
U S APPLICATION NO (IF KNOWN, SEE 37 CFR)	To be assigned 10/089776

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/06743	28 September 2000	29 September 1999

TITLE OF INVENTION
HIGH SENSITIVITY IMMUNOASSAY METHOD

APPLICANT(S) FOR DO/EO/US
Kei TASHIRO; Tasuku HONJO; Masaya IKEGAWA; and Kazuko MATSUMOTO

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a ☒ is attached hereto
   b ☐ has been previously submitted under 35 U S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S C. 371 (c)(3))
   a ☐ are attached hereto (required only if not communicated by the International Bureau).
   b ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U S.C. 1.821 - 1.825.
20. ☒ A second copy of the published international application under 35 U S C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U S C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information.

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JC10 Rec'd PCT/PTO 29 MAR 2002

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/089776 To be assigned	PCT/JP00/06743	29288.5600

24. The following fees are submitted:	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO . . . **$890.00**	
☐ International preliminary examination fee (37 CFR 1 482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**	
☐ International preliminary examination fee (37 CFR 1 482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
☐ International preliminary examination fee (37 CFR 1 482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	16 - 20 =	0	x $18.00	**$0.00**	
Independent claims	2 - 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,020.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$1,020.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,020.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3 31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,020.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,020.00** to cover the above fees is enclosed

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **19-2814** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Michael K. Kelly
SNELL & WILMER, LLP
One Arizona Center
400 E. Van Buren Street
Phoenix, AZ 85004-2202
P: (602) 382-6291
F: (602) 382-6070

Michael K. Kelly
SIGNATURE

Michael K. Kelly
NAME

32,848
REGISTRATION NUMBER

3/29/2002
DATE

DESCRIPTION

HIGH SENSITIVITY IMMUNOASSAY METHOD

TECHNICAL FIELD

The present invention relates to a time-resolved fluoroimmunoassay (TR-FIA) method for detecting cytokines in a biological fluid sample, and in particular to an assay method for highly sensitively detecting cytokines in a biological fluid sample by utilizing a fluorescent europium complex.

BACKGROUND ART

The concentration of free cytokines or chemokines present in a normal biological fluid such as human plasma is near or below the detection limit of conventional ELISA assays. For example, it has been reported that a conventional ELISA assay whose detection limit is about 6 picomols (pM) cannot detect IL-8 from within normal human plasma (Leonard et al. (Document 1)). Enhancement of measurement sensitivity and reduction of the non-specific background associated with the biological fluid sample are chief problems to be solved in order to attain accurate measurement of chemokine concentration in a

biological fluid sample.

In recent years, a time-resolved fluoroimmunoassay method which utilizes a europium complex has been developed, and is being used in clinical applications (Kropf et al., (Document 2)). The radiation wavelength (615 nm) of a free, complexed europium ion (Eu^{3+}) is not influenced by the excitation wavelength (340 nm) or by a transient background fluorescence (350 to 600 nm) associated with a certain type of protein, which is convenient. One type of analysis method which is based on this principle is commercialized as DELFIA (dissociation-enhanced lanthanoid fluoroimmunoassay; Pharmacia), and is utilized in assays of TNFα and IL-6. However, DELFIA has not been successful in accurately measuring the concentration of such cytokines in plasma (Ogata et al. (Document 3)).

Recently, a group led by Matsumoto has developed a 4,4'-bis(1",1",2",2",3",3",-heptafluoro-4",6",-hexanedion-6"-yl)-sulpho-o-terphenyl(BHHCT)-Eu^{3+} complex as a labeling compound. This complex is capable of directly binding to proteins, and allows for highly

sensitive analysis via a time-resolved type fluorescence measurement (Yuan et al.('97)(Document 4) and Yuan et al.('98)(Document 5)). BHHCT has a β -diketone structure, and has a binding stability constant with respect to Eu^{3+} as high as $10^{10}M^{-1}$. A resultant Eu^{3+} complex exhibits quite excellent properties, as evidenced by a lifetime which exceeds 400 microseconds (μs), and absorption and emission wavelength maximals of 330 nm and 615 nm. This complex has been indicated to be useful for the detection of α -fetoprotein (Yuan et al.('98)(Document 5) and immunoglobulin E (IgE) Yuan et al.('97)(Document 4)), which are tumor markers in human plasma. However, no instances are known in which such an Eu^{3+} complex has been applied to the detection of cytokines in a biological fluid sample.

Stromal cell-derived factor-1 (SDF-1) is a cytokine belonging to the chemokine family, which was first cloned from a stromal cell line in 1993 (Tashiro et al. (Document 6)). SDF-1 is a chief ligand for a CXCR4 receptor (Bleul et al. (Document 7) and Oberlin et al.(Document 8)). This receptor is known to function as a CD4 co-receptor for a subgroup of human immunodeficiency virus type 1 (HIV-1). Furthermore,

recent study has shown that polymorphism of the SDF-1 gene is involved in slowing of the progression of acquired immunodeficiency syndrome (AIDS) (e.g., Winkler et al. (Document 9) and Martin et al. (Document 10)). However, its functional mechanism admits of several theories, and is yet to be established.

It has also been pointed out that SDF-1 plays an essential role in embryogenesis of the hematopoietic, cardiovascular, and nervous systems (e.g., Zou et al. (Document 11) and Tachibana et al. (Document 12)). On the other hand, many of the biological functions of SDF-1 in adult tissue are still unknown.

As described above, it is extremely important for advancement of the understanding of SDF-1 to develop a technique for accurately quantifying and monitoring SDF-1 in a biological fluid sample. It is needless to say that an accurate measurement method in biological fluid samples would similarly make academic and clinical contributions in other chemokines and cytokines as well. From this perspective, an assay method for detecting cytokines with a higher sensitivity is desired.

DISCLOSURE OF THE INVENTION

The present invention aims to solve the aforementioned problems, and provides a method for detecting cytokines in a biological fluid sample with a higher sensitivity and ease.

According to the present invention, there is provided a time-resolved fluoroimmunoassay (TR-FIA) method for detecting a cytokine in a biological fluid sample, comprising:

forming a composite in which (a) a first antibody including a portion bound to a solid phase and a region bindable to a cytokine; (b) the cytokine; (c) a second antibody including a region bindable to the cytokine and a portion to which biotin is bound; (d) a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and (e) the lanthanoid metal ion are bound, the composite being formed on the solid phase; and

measuring fluorescence of the fluorescent structural portion which has been complexed with the lanthanoid metal ion,

wherein the fluorescent structural portion is

represented by General Formula (I):

$$-R-Ar-C(=O)-CH_2-C(=O)-C_nF_{2n+1}-X \qquad (I)$$

(where R is a residue which is a functional group capable of forming a covalent bond with a protein; Ar is a hydrocarbon group having a conjugated double bond system; n is an integer equal to or greater than 1; and X is a fluorine atom or a group represented by General Formula (II):

$$-C(=O)-CH_2-C(=O)-Ar-R- \qquad (II).$$

In one embodiment of the present invention, the lanthanoid metal ion may be europium.

In one embodiment of the present invention, the fluorescent structural portion may be represented by General Formula (III):

$$-R-Ar-(C(=O)-CH_2-C(=O)-C_nF_{2n+1})_2 \qquad (III)$$

(where R, Ar, and n have the same definitions as above).

In one embodiment of the present invention, the fluorescent structural portion may be 4,4'-bis(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl)-sulpho-o-terphenyl.

In one embodiment of the present invention, 10 to 60 units of the fluorescent structural portion may be present per molecule of streptoavidin or avidin in the conjugate.

In one embodiment of the present invention, the step of measuring fluorescence may be performed without allowing the composite formed on the solid phase to dissociate.

In another embodiment of the present invention, the step of measuring fluorescence may be performed after allowing the composite formed on the solid phase to dissociate.

In one embodiment of the present invention, the cytokine may be a cytokine belonging to the chemokine family.

In one embodiment of the present invention, the cytokine may be a CXC chemokine.

In one embodiment of the present invention, the cytokine may be stromal cell-derived factor-1 (SDF-1).

Alternatively, in one embodiment of the present invention, the cytokine may be a cytokine which exist as a soluble factor in blood circulation and has a biological activity in a minuscule amount.

Alternatively, in one embodiment of the present invention, the cytokine may be a granulocyte-macrophage-colony stimulating factor (GM-CSF) or interleukin 2 (IL-2).

In one embodiment of the present invention, the biological fluid sample may be plasma or whole blood.

In one embodiment of the present invention, a step of diluting the biological fluid sample with a buffer solution used for sample dilution may be further comprised before the step of forming the composite, and the buffer solution used for sample dilution may be 0.01 to 0.1 M

tris-hydrochloric acid whose pH is 7.3 to about 8.3, the buffer solution containing 0.1 to 0.3% of bovine serum albumin, 0.05 to 0.2% of sodium azide, and 0.5 to 1.5% of sodium chloride.

In one embodiment of the present invention, a step of subjecting the biological fluid sample to a heat treatment under non-denaturing temperature conditions for the cytokine may be further comprised before the step of forming the composite.

In one embodiment of the present invention, a step of washing the composite formed on the solid phase with a buffer solution used for washing may be further comprised before the step of measuring fluorescence, and the buffer solution used for washing the composite may be 0.01 to 0.1 M tris-hydrochloric acid whose pH is 8.5 to about 9.5, the buffer solution containing 0.01 to 0.1% polyoxyethylenesorbitan monolaurate.

In one embodiment of the present invention, the solid phase may be a microtiter plate having an IgG adsorption ability of 50 to 200 ng/cm^2.

Moreover, according to the present invention, there is provided a kit for a time-resolved fluoroimmunoassay (TR-FIA) method for detecting a cytokine in a biological fluid sample, comprising: a first antibody including a portion bound to a solid phase and a region bindable to a cytokine; a second antibody including a region bindable to the cytokine and a portion to which biotin is bound; a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and the lanthanoid metal ion,

wherein the fluorescent structural portion is represented by General Formula (I):

$$-R-Ar-C(=O)-CH_2-C(=O)-C_nF_{2n+1}-X \qquad (I)$$

(where R is a residue which is a functional group capable of forming a covalent bond with a protein; Ar is a hydrocarbon group having a conjugated double bond system; n is an integer equal to or greater than 1; and X is a fluorine atom or a group represented by General Formula (II):

$$-C(=O)-CH_2-C(=O)-Ar-R- \qquad (II).$$

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a is a graph illustrating a calibration curve for SDF-1. A reference SDF-1 was measured by using a TR-FIA method described in Example 2. The data indicate average values of triplicate measurements.

Figure 1b is a graph illustrating a similar calibration curve as in Figure 1a with a particular focus on the measurements in a low concentration range. The line in the graph is as follows: $Y = 1.3X + 1.2$ ($\times 10000$ a.u.); $r = 0.995$. The data indicate average values of triplicate measurements.

Figure 1c is a graph illustrating measurement results of CXCR4 expression on the EL-4 cell surface according to a protocol described in Example 3 as a way of monitoring the biological activity of SDF-1. The percentage decrease in a mean fluorescence intensity (MFI) was calculated based on comparison with controls which were incubated without human SDF-1β. The data represent medians selected from three runs of a series of experiments.

Figure **1d** is a graph illustrating measurement results of various chemokines for evaluating the specificity of TR-FIA with respect to SDF-1. The data indicate average values of triplicate measurements.

Figure **2** is a graph illustrating a comparison between TR-FIA and DELFIA with respect to SDF-1. On the left-hand side of Figure **2** are shown measurement results of a reference solution of human SDF-1β by DELFIA and TR-FIA systems by employing the same combinations of a capture antibody and a detection antibody as those employed in Example 2. On the right-hand side of Figure **2** are shown results of endogenous SDF-1 concentrations within plasma samples as obtained by the two systems. The samples shown on the right-hand side of Figure **2** have no reference SDF-1 added thereto. The data on the right-hand side of Figure **2** and the data shown in Table 1 represent measurement results for different samples. The data indicate average values of triplicate measurements.

Figure **3a** is a graph illustrating the influences of anticoagulants and protease inhibitors on the SDF-1 measurement by TR-FIA. Plasma samples were treated with

EDTA (1 mg/ml); heparin (30 IU/ml); a citrate (sodium citrate 0.38%); or EDTA (1 mg/ml) containing aprotinin (1 μg/ml). The block bars and the hatched bars represent measurement results for two different samples. The data indicate average values of triplicate measurements.

Figure **3b** is a graph illustrating influences of preliminary heating of plasma samples on the SDF-1 measurement by TR-FIA. Plasma samples were previously incubated at 55℃ for 30 minutes before the assay, or directly used for measurement without any heating. The plasma samples were obtained from 24 healthy Japanese volunteers. The data indicate average values of duplicate measurements.

Figure **3c** is a graph illustrating influences of plasma sample dilution on the SDF-1 measurement by TR-FIA. Each sample was diluted in Buffer Solution 4. The plasma samples were obtained from 5 healthy Japanese volunteers. The data indicate average values of triplicate measurements.

Figure **4a** is a graph illustrating influences of blood cells on an ELISA quantification of IL-8, as a

control for SDF-1. After IL-8 was added to plasma samples, cell pellets or plasma was mixed therewith. After incubation at 37℃ for 15 minutes, the soluble IL-8 within the plasma was quantified. Blank squares represent reference samples which were not mixed with cell pellets or plasma; black circles represent samples which were mixed with plasma; and blank circles represent samples which were mixed with cell pellets. The data indicate average values of quadruplicate measurements.

Figure **4b** is a graph illustrating influences of blood cells on an ELISA quantification of MCP-1, as a control for SDF-1. After MCP-1 was added to plasma samples, cell pellets or plasma was mixed therewith. After incubation at 37℃ for 15 minutes, the soluble MCP-1 within the plasma was quantified. The symbols are similar to those in Figure **4a**. The data indicate average values of quadruplicate measurements.

Figure **4c** is a graph illustrating influences of blood cells on a TR-FIA quantification of SDF-1. After SDF-1 was added to plasma samples, cell pellets or plasma was mixed therewith. After incubation at 37℃ for 15 minutes, the soluble SDF-1 within the plasma was

quantified. The symbols are similar to those in Figure 4a. The data indicate average values of quadruplicate measurements.

Figure 5 is a graph illustrating SDF-1 levels in human plasma from 36 healthy Japanese volunteers. All plasma samples were subjected to a heat treatment at 55℃ for 30 minutes before the assay. The data indicate average values of triplicate measurements from two separate measurings.

Figure 6 is a graph illustrating the influences of IgG depletion due to protein G-sepharose on human plasma samples. Plasma samples from 7 healthy Japanese volunteers were incubated on ice with protein G-sepharose for 30 minutes and centrifuged, and the SDF-1 amount in supernatants were measured. Hatched bars and black bars represent unheated samples and heated samples (55℃ for 30 minutes), respectively.

Figure 7 is a graph illustrating a calibration curve for GM-CSF. A reference GM-CSF was measured by a TR-FIA method. The data indicate average values of triplicate measurements.

Figure 8 is a graph illustrating a calibration curve for IL-2. A reference IL-2 was measured by a TR-FIA method. The data indicate average values of triplicate measurements.

BEST MODES FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention will be described in more detail.

The method of the present invention is based on a time-resolved fluoroimmunoassay (TR-FIA) technique. A "time-resolved fluoroimmunoassay" refers to an assay method which labels a measurement subject with a fluorescent compound that is capable of radiating long-life fluorescence, e.g., a lanthanoid metal ion complex according to the present invention, through an immunological reaction, and taking time-resolved type measurements of a fluorescent signal from the labeled subject after the background fluorescence having a shorter life time has disappeared.

The method according to the present invention is particularly suitable for a highly sensitive detection

of cytokines in a biological fluid sample. A "biological fluid sample" refers to liquid matter which is collected from a living animal, preferably a mammal, and in particular a human. Representative examples thereof include blood (i.e., whole blood) and its fractions or plasma and serum, as well as cerebral spinal fluid, bile, amniotic fluid, pleural fluid, ascites, tracheobronchial secretion, marrow fluid, milk, lacrimal fluid, nasal discharge, endocardial fluid, intra-articular fluid, saliva, semen, urine, and the like. Furthermore, biological fluid samples may also include supernatants of cultured cells of animal origin and the like. In the method according to the present invention, remarkable effects can be provided when using whole blood, plasma, serum, or cerebral spinal fluid, and in particular when using whole blood or plasma. For convenience, a biological fluid sample, as used herein, includes both a biological fluid itself and a liquid sample which has been subjected to a treatment such as dilution in a carrier which is suitable for the biological fluid.

A "cytokine" refers to a proteinaceous chemical substance which is responsible for information transmission between cells in a living organism. For each

individual cytokine, a characteristic receptor is expressed on the surface of a target cell. Binding to such a receptor results in the manifestation of physiological activities such as cell growth and differentiation. A group of cytokines collectively referred to as "hematopoietic factors", which induce the differentiation and growth of blood cells, include colony stimulating factors (CSFs) including granulocyte-macrophage-colony stimulating factors (GM-CSFs), stem cell factors, erythropoietin, thrombopoietin, and the like. Interleukins which control lymphocytes include IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-18, and the like. A group of cytokines collectively referred to as "growth factors" include the TGF-β family, the EGF family, the FGF family, the IGF family, the NGF family, blood platelet-derived growth factors (PDGFs), hepatic cell growth factors (HGFs), vascular endothelial cell growth factors (VEGFs), and the like. A group of cytokines collectively referred to as "tumor necrosis factors" include TNF-α, TNF-β, and the like. A group of cytokines collectively referred to as "interferons" include INF-α, INF-β, INF-γ, and the like. Other known cytokines include endotheline, glial cell-derived neurotrophic factors (GDNFs), and the like. A group of cytokines which

impart chemotaxis to any one of functionally mature blood cells are particularly referred to as chemokines. Depending on the conserved cysteine location at their N-terminus regions, chemokines are classified into four categories: CC, CXC, C, or CXXXC.

The detection subject for the method according to the present invention may be any one of the aforementioned cytokines. Furthermore, any newly discovered members of any one of the aforementioned groups of cytokines, or any newly discovered cytokines which do not belong to any one of the aforementioned groups of cytokines, may also be detection subjects for the method according to the present invention. In particular, the method according to the present invention is applicable to cytokines which exist as soluble factors in blood circulation, have a biological activity in minuscule amounts, and are involved in various pathologies.

An example of a detection subject for the method according to the present invention may be cytokines belonging to the aforementioned chemokine family, and in particular CXC chemokines, but is not necessarily limited to such categories. A most preferable example of a

detection subject for the method according to the present invention is SDF-1.

In the method according to the present invention, in order to selectively capture and label a desired cytokine in a biological fluid sample, a composite containing that cytokine is formed on a solid phase. Specifically, a cytokine-containing composite is formed from the following components on an appropriate solid phase:

(a) a first antibody including a portion bound to a solid phase and a region bindable to a cytokine;

(b) the cytokine;

(c) a second antibody including a region bindable to the cytokine and a portion to which biotin is bound;

(d) a conjugate including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion; and

(e) the lanthanoid metal ion.

Hereinafter, the respective components will be described.

As the "solid phase", a solid substance of any

shape and material may be used so long as it allows an antibody to bind thereto and does not hinder the formation of the aforementioned conjugate and the fluorescence measurement (described later). For convenience of performing the assay method, a microtiter plate of a multiwell type is typically used, but any other configuration may be used such as a column filled with beads (where the material of the beads may be sepharose, agarose, etc., although not limited thereto). According to the present invention, a microtiter plate which exhibits an intermediate protein adsorption ability may be particularly suitable. As used herein, an "intermediate protein adsorption ability" refers to a property which exhibits typically about 50 to about 200 ng/cm^2, preferably about 15 to 150 ng/cm^2, and more preferably about 90 to about 120 ng/cm^2 when immunoglobulin G (IgG) is adsorbed as a reference protein. The material of the microtiter plate may preferably be polystyrene, although not limited thereto.

Component (a), or the "first antibody", is an antibody which exists in a bound state to the aforementioned solid phase and which is capable of binding to a desired cytokine through an antigen-antibody

reaction. In this sense, the first antibody is also referred to as a "capture antibody". In the present specification, an "antibody" is meant to include an immunoglobulin (Ig) and an immunoglobulin-derived molecule of any type, e.g., a polyclonal antibody, a monoclonal antibody, Fab, $(Fab)_2$, or a chimeric antibody. The term "antibody" is used with a broad meaning, and so long as being capable of binding to a cytokine in a manner similar to an immunoglobulin, even includes a receptor having that cytokine as a ligand. An example of a preferable antibody is a polyclonal antibody or a monoclonal antibody. Antibodies to various cytokines are commercially available from, for example, R&D System Inc. (Minnesota, US), Dako Immunoglobulins a/s (Denmark), PharMingen (California, US), Southern Biotechnology Associates (Alabama, US), and the like. Alternatively, an antibody to a desired cytokine can be created by using usual methods such as animal immunization or hybridoma techniques.

Binding to the solid phase can be achieved following usual methods, e.g., by directly coating the first antibody onto a microtiter plate. The "portion bound to a solid phase" of the first antibody typically

refers to an Fc region of an antibody which is partially adsorbed to a solid phase, although not limited thereto. For example, a bifunctional linker molecule which is capable of binding to the solid phase and to a portion of the antibody can be used.

Component (b), or a desired cytokine which is present in a biological fluid sample is immobilized to the solid phase, typically by binding to the first antibody. The cytokine does not need to be in a free state to be in contact with the first antibody. For example, the cytokine may bind to the first antibody after binding to the second antibody (described later). Thus, the conjugate formation according to the present invention is not limited with respect to the order of binding of the respective components.

The inventors found that it is essential for highly sensitive cytokine detection that the biological fluid sample containing a desired cytokine is diluted to an appropriate concentration in an appropriate buffer solution before being exposed to an antibody which is capable of binding to that cytokine. The dilution ratio by the biological fluid sample buffer solution may

typically be about 1 : 1 to about 1 : 30, preferably about 1 : 2.5 to about 1 : 20, and more preferably about 1 : 5 to about 1 : 15, as represented on a volume basis of (biological fluid sample: buffer solution). The optimum value of the dilution ratio may vary depending on the kind of biological fluid sample and the kind of cytokine, etc., and further on the composition of the buffer solution used for sample dilution.

An appropriate buffer solution used for sample dilution is an alkalescent buffer solution which is composed of tris(hydroxymethyl)aminomethane (abbreviated as "Tris") and an inorganic acid, and typically a tris-hydrochloric acid, whose pH is typically about 7.0 to about 8.6, preferably about 7.3 to about 8.3, and more preferably about 7.5 to about 8.1, and whose concentration is typically about 0.005 to about 0.2 mol(M), preferably about 0.01 to about 0.1 M, and more preferably about 0.025 to about 0.075 M.

The buffer solution used for sample dilution further contains appropriate amounts of a plasma protein component and salts. The plasma protein component is typically serum albumin and preferably bovine serum

albumin (BSA), whose concentration is typically about 0.05 to about 0.5%, preferably about 0.1 to about 0.3%, and more preferably about 0.15 to about 0.25%. The salts are typically sodium azide (NaN_3) and sodium chloride (NaCl). The concentration of NaN_3 may typically be about 0.02 to about 0.4%, preferably about 0.05 to about 0.2%, and more preferably about 0.05 to about 0.15%. The concentration of NaCl may typically be about 0.2 to about 3%, preferably about 0.5 to about 1.5%, and more preferably about 0.6 to about 0.12%.

It will be appreciated that the composition of the buffer solution used for sample dilution is not limited to the aforementioned conditions, and admits of various modifications that come easy to those skilled in the art. For example, it is possible to replace part or whole of the aforementioned sodium salts with other alkaline metal salts or corresponding alkaline earth metal salts. The optimum values of the pH of the buffer solution used for sample dilution and the concentrations of the respective components may vary depending on the kind of cytokine which is the detection subject, and may also depend on the dilution ratio of the biological fluid sample. Such optimization can be attained within the bounds of the

usual condition setting processes by those skilled in the art.

Component (c), or the "second antibody", includes a region bindable to the cytokine so as to capture a desired cytokine in a sandwiching fashion with the first antibody. It is desirable that the first antibody and the second antibody are anti-peptide antibodies which recognize different sites (i.e., different epitopes) of the same cytokine molecule without interfering with each other. Therefore, it is essential that the first antibody and the second antibody make a suitable combination in terms of binding ability with the desired cytokine. For example, suitable combinations can be selected from among multiple lots of polyclonal antibodies which are obtained by immunizing an appropriate animal with the full-length cytokine or a fragment of that cytokine which is known or predicted to include a plurality of epitopes. Alternatively, suitable combinations can be selected from among a plurality of monoclonal antibodies which recognize different epitopes. Such a selection can be achieved without particular difficulties through a preliminary experiment which involves preparing a reference solution of cytokine and performing a usual

ELISA method with respect to combinations of antibodies to be considered, for example.

The second antibody may further include a portion to which biotin is bound so as to enable detection of the cytokine through fluorescence measurement. In this sense, the second antibody is also referred to as a "detection antibody". Biotin is a vitamin which is also referred to as vitamin H or coenzyme R, and is capable of forming an amide bond with an amino group such as a peptide. The second antibody can be prepared by biotinating and purifying an antibody to the cytokine which is the detection subject following usual methods. The "portion to which biotin is bound" of the second antibody refers to biotin itself as well as the part of the antibody to which biotin is bound (typically the Fc region). If necessary, biotin and a portion of the antibody may be linked by using a bifunctional linker molecule which is capable of binding to both.

As used herein, the expression "second antibody" does not necessary refer to a single molecule, but may represent any structural unit that fulfills the required functions (i.e., the function of being able to bind to

a cytokine through an antigen-antibody reaction or a ligand-receptor bond, and the function of carrying biotin). The same also applies to the aforementioned "first antibody". For example, a combination of an antibody to a desired cytokine and a biotinated anti-IgG antibody which is capable of binding to this anti-cytokine antibody can be employed in the present invention. In this case, the combination of the anti-cytokine antibody and the biotinated anti-IgG antibody is collectively referred to as the "second antibody". A biotinated anti-IgG antibody is convenient because of its versatility. In the case where an antibody to a desired cytokine has resistance against a biotination reaction for some reason, the use of a combination with a biotinated anti-IgG antibody may be useful. On the other hand, from the perspective of simplifying the assay procedure and maximizing the cytokine detection sensitivity, it is preferable to employ a single molecule as the second antibody.

Component (d), or a "conjugate" is any structural unit including streptoavidin or avidin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion, and is typically a molecule in which

streptoavidin or avidin and the fluorescent structural portion are directly or indirectly linked via a covalent bond. Streptoavidin is generally well-known as a protein produced by Actinomycetes and having a molecular weight of about 60,000, and strongly binds to biotin by nature. In the present invention, however, "streptoavidin" is not limited to those of any particular microbial origin, but may include corresponding proteins of any other microbial origin, as well as modifications thereof, so long as its binding ability with biotin is substantially retained. Avidin is generally well-known as a protein having a molecular weight of about 70,000 contained in egg white, and also strongly binds to biotin by nature. In the present invention, "avidin" is not necessarily limited to natural egg white protein, but may include modifications thereof so long as its binding ability with biotin is substantially retained.

As will be seen from the aforementioned principles, the method according to the present invention can also be carried out by employing, instead of component (c), an antibody including a region bindable to a cytokine and a portion to which streptoavidin or avidin is bound; and employing, instead of component (d), a conjugate which

includes biotin and a fluorescent structural portion capable of being complexed with a lanthanoid metal ion.

The fluorescent structural portion of the conjugate of component (d) that is capable of being complexed with a lanthanoid metal ion is a partial structure which be obtained by allowing a corresponding fluorescent compound to react so as to be directly or indirectly linked via a covalent bond with streptoavidin or avidin. The fluorescent structural portion is represented by General Formula (I) below:

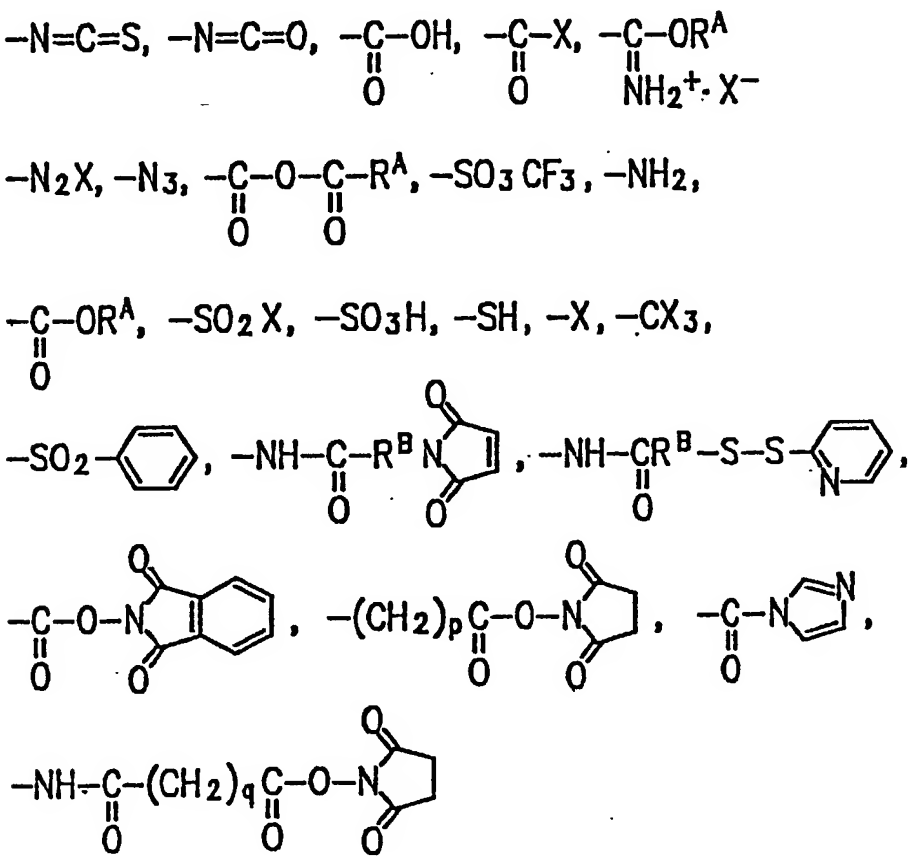
$$-R-Ar-C(=O)-CH_2-C(=O)-C_nF_{2n+1}-X \qquad (I)$$

(in the formula, R represents a residue which is a functional group capable of forming a covalent bond with a protein; Ar represents a hydrocarbon group having a conjugated double bond system; n is an integer equal to or greater than 1; and X is a fluorine atom or a group represented by General Formula (II):

$$-C(=O)-CH_2-C(=O)-Ar-R- \qquad (II)$$

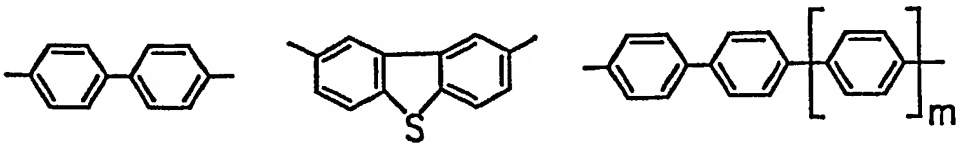
In the above general formulae, the "functional

group which is capable of forming a covalent bond with a protein", which defines the residue R, refers to any organic functional group that is capable of forming a covalent bond by reacting with any reactive group (typically an amino group, a carboxyl group, and a hydroxyl group) included in an amino acid residue within the protein. Examples of such functional groups include the following groups:

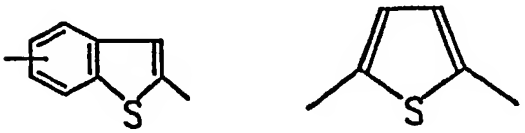


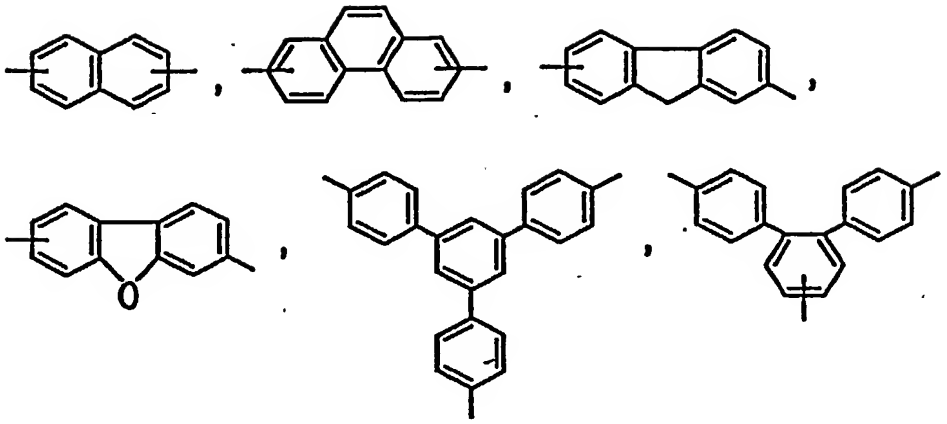
(where X is selected from a halide atom, $-OSO_2CH_3$, $-OSO_2F$, $-OSO_2CF_3$, $-OSO_2C_4F_9$, or $-OSO_2PhCH_3$-p (where Ph represents a phenyl group); R^A is selected from an alkyl group, an alkenyl group, an aryl group, or an aralkyl group; R^B is selected from an alkylene group, an alkenylene group, an arylene group, or an aralkylene group; p is 0 to 5; and q is 2 to 10).

In the above general formulae, the "hydrocarbon group having a conjugated double bond system", which defines Ar, is a hydrocarbon group having at least three conjugated double bonds, and is typically a divalent or trivalent aromatic hydrocarbon group having at least one phenyl ring. The upper limit of the number of carbons in the hydrocarbon group is typically about 50 or less, and preferably about 30 or less, although not particularly limited thereto. Herein, one or more carbon may be substituted by a hetero atom (e.g., an oxygen or sulfur atom). Examples of the hydrocarbon group Ar include the following groups:



(where m is an integer of 1 to 6)





Preferably, the hydrocarbon group Ar is trivalent, and the fluorescent structural portion is represented by General Formula (III):

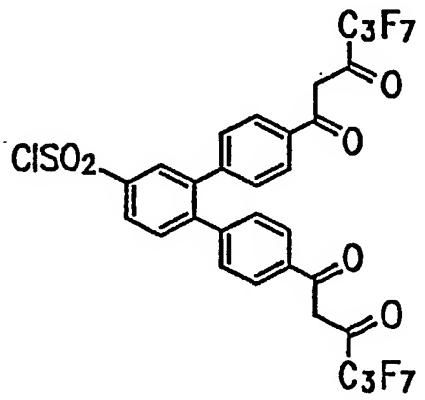
$$-R-Ar-(C(=O)-CH_2-C(=O)-C_nF_{2n+1})_2 \qquad (III)$$

Herein, a more preferable example of Ar is o-terphenyl which binds to two β-diketone groups at the 4,4' positions. Another similarly preferable example of Ar is a trivalent aromatic hydrocarbon group which can cause two β-diketone groups to be positioned at similar locations to, or at substantially the same spatial distance as, the locations of the β-diketone groups associated with o-terphenyl.

In the above general formulae, n is an integer

of 1 or more, typically 1 to 6, and preferably 2 to 4.

In the present invention, a particularly preferable fluorescent structural portion is 4,4'-bis(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl)-sulpho-o-terphenyl. This is obtained from a corresponding fluorescent compound 4,4'-bis(1",1",1",2",2",3",3"-heptafluoro-4",6"-hexanedion-6"-yl)-chlorosulpho-o-terphenyl (abbreviated as "BHHCT"). The structural diagram of BHHCT is shown below:



A desired fluorescent compound which gives the aforementioned fluorescent structural portion can be synthesized by utilizing routine organic synthesis reactions. Typically, it can be synthesized by following a procedure consisting of the following two steps:

(First step) A claisen condensation reaction between an acetylated aromatic compound and perfluorocarboxylate ester is carried out in an appropriate solvent in the presence of a basic catalyst (e.g., sodium methylate), thereby producing a β-diketone compound in which the CH_3- of an acetyl group has been perfluorocarbonylated.

(Second step) A functional group which is capable of forming a covalent bond with a protein is introduced into the β-diketone compound. For example, hydrogens of the aromatic ring are substituted by chlorosulfonyl groups ($ClSO_2$-) through a chlorosulfonylation reaction using chlorosulfuric acid. After the respective steps, purification such as recrystallization or precipitation can be performed as necessary.

The resultant fluorescent compound is allowed to react with a protein under appropriate conditions, depending on the kind of the functional group which was introduced during the aforementioned second step, thereby giving the fluorescent structural portion of interest. For example, a chlorosulfonyl group easily forms an amide

with an amino acid within a protein under basic reaction conditions.

In the present invention, the conjugate of component (d) can be prepared by directly labeling streptoavidin or avidin with a fluorescent compound. Alternatively, the conjugate of component (d) can be prepared by first allowing streptoavidin or avidin to conjugate to another protein (e.g., bovine serum albumin) and then further labeling it. The conjugation between the streptoavidin or avidin and another protein can be achieved following usual methods, e.g., by a cross-linking reaction using glutaraldehyde.

The labeling reaction for the protein with a fluorescent compound can be carried out typically by dissolving the protein in a buffer solution which has been adjusted to an appropriate pH for the reaction (e.g., approximately pH 9 in the case of chlorosulfonylation), and adding thereto a fluorescent compound which has been dissolved in an appropriate solvent (e.g., ethanol or dimethylformamide in such an amount as to achieve a desired molar ratio. By adjusting the molar ratio of the fluorescent compound to the protein and the concentration

of the solution containing the fluorescent compound, it is possible to control the ratio (also referred to as the "conjugation ratio") of the fluorescent compound which conjugates to each molecule of the protein. The conjugation ratio corresponds to the number of units of the fluorescent structural portion which are present per molecule of streptoavidin or avidin in the conjugate according to the present invention. The conjugation ratio may typically be 5 to 100 units, and preferably 10 to 60 units. If the conjugation ratio is too small, a sufficiently high cytokine detection sensitivity may not be obtained. On the other hand, too high a conjugation ratio may not make for the improvement in the detection sensitivity.

The composite formation on a solid phase in the method according to the present invention is accomplished as component (e) of a lanthanoid metal ion complexes with the aforementioned fluorescent structural portion. Examples of lanthanoid metal ions include europium (Eu), samarium (Sm), terbium (Tb), and dysprosium (Dy). Europium (Eu) is preferable. The lanthanoid metal ion is previously complexed with the conjugate of component (d) and utilized for the composite formation

in that form. In other words, usually the fluorescent structural portion has already become a complex retaining Eu^{3+} at the time when the conjugation with streptoavidin or avidin or biotin is formed. However, this does not exclude the opposite procedure.

The inventors found that it is essential for a high-sensitivity cytokine detection that the composite, which has been thus formed on a solid phase, be adequately washed with an appropriate buffer solution prior to the fluorescence measurement. Herein, an appropriate buffer solution used for washing the composite is an alkaline buffer composed of Tris and inorganic acids, and is typically tris-hydrochloric acid whose pH is typically about 8.2 to about 9.8, preferably about 8.5 to about 9.5, and more preferably about 8.7 to about 9.4, and whose concentration is typically about 0.005 to about 0.2 M, preferably about 0.01 to about 0.1 M, and more preferably about 0.025 to about 0.075 M.

The buffer solution used for washing the composite further contains an appropriate amount of nonionic surfactant having a protein solubilizing ability. The nonionic surfactant is typically polyoxyethylenesorbitan

monolaurate, and preferably a polyoxyethylenesorbitan monolaurate which is commercially available under the product name "Tween (registered trademark) 20" (molecular weight: about 1200). Other nonionic surfactants which have substantially the same properties as those of Tween (registered trademark) 20 (e.g., a hydroxy value about 95 to about 115; a saponification value of about 35 to about 55; and an HLB (hydrophilicity-hydrophobicity balance) of about 15 to 18)) can also be preferably used. The concentration of the nonionic surfactant is typically about 0.005 to about 0.2%, preferably about 0.01 to about 0.1%, and more preferably about 0.025 to about 0.075%.

It will be appreciated that the composition of the buffer solution used for washing the composite is not limited to the aforementioned conditions, and various modifications that are easy to those skilled in the art are permitted. The maximum values of pH, the concentrations of the respective components may vary depending on the kind of cytokine to be detected. Such optimization can be achieved within the scope of the usual condition setting process by those skilled in the art.

Hereinafter, a typical example of a procedure for

the composite formation on a solid phase according to the method of the present invention will be described.

1) A solution of the first antibody which has been diluted in an appropriate buffer solution used for coating is applied on a solid phase (e.g., in a well of a 96-well microtiter plate), and the first antibody is immobilized on the solid phase through incubation. As the buffer solution used for coating, a phosphate buffer solution containing an appropriate amount of NaCl may be employed, for example. Typically, the incubation conditions are about 2 to 6℃ for about 20 hours or more.

2) Next, the surface of the solid phase which has been coated with the first antibody is washed several times with a buffer solution used for washing. As the buffer solution used for washing, for example, alkalescent tris-hydrochloric acid may be employed, and an appropriate amount of a nonionic surfactant having a protein solubilizing ability may be added as necessary. After washing, the coated solid phase is preserved at a low temperature of about -20℃ until immediately before it is used for an assay.

3) As described above, the biological fluid sample containing a cytokine which is the detection subject is preferably previously diluted to an appropriate level with a buffer solution used for sample dilution. The biological fluid sample, and if necessary a reference solution of the cytokine, is applied to the coated solid phase and incubated. Typically, the incubation conditions are about 35 to 39℃ for about 40 minutes to about 2 hours. After incubation, the surface of the solid phase is washed several times with a buffer solution used for washing, similarly as above.

4) Thereafter, a solution of the second antibody which has been diluted in an appropriate buffer solution is applied to a solid phase and incubated. Herein, it is preferable to employ the same buffer solution used for sample dilution as that described above. The incubation conditions are similar to those in the aforementioned incubation for the biological fluid sample. After incubation, the surface of the solid phase is washed several times with a buffer solution used for washing, similarly as above.

5) The conjugate is mixed with a solution of a salt of

a lanthanoid metal ion so as to allow a fluorescent complex portion to be formed. After being diluted in an appropriate solvent, the complexed conjugate is applied to a solid phase and incubated. The incubation conditions are similar to those in the aforementioned incubations for the biological fluid sample and the second antibody. After incubation, the composite which has been formed on the solid phase is washed several times with an appropriate buffer solution used for composite washing, in the aforementioned manner.

Next, the composite containing a lanthanoid complex which has been obtained in the aforementioned manner is subjected to a time-resolved fluorescence measurement in a solid or liquid phase. Apparatuses for this fluorescence measurement are commercially available. Typically, the measurement conditions are: delay time of about 0.2 to about 0.3 milliseconds (ms); a window time of about 0.2 to about 0.6 ms; a flash rate of about 0.5 to about 1.5 ms; an excitation wavelength of 337.1 nm (wavelength of a nitrogen laser); and a measurement wavelength of 615 nm.

In the case of a solid phase fluorescence

measurement, the solid phase bearing the aforementioned composite can be subjected to the fluorescence measurement conditions as it is. In the case of a liquid phase fluorescence measurement, the composite is treated with an appropriate dissociation solution to allow any structural units containing the fluorescent complex portion to break free into the solution, and this solution is subjected to the fluorescence measurement conditions. The dissociation is typically a weak-basic aqueous solution containing trialkylphosphinoxide and an anionic surfactant. As an example of a dissociation solution, an aqueous solution of sodium hydrogen carbonate ($NaHCO_3$) containing tri(n-octyl)phosphinoxide (TOPO) and sodium dodecyl sulfate (SDS) may be used. By incubating the solid phase bearing the aforementioned composite at about 45 to 55℃ for about 40 minutes to about 2 hours, the conjugation with the streptoavidin or avidin or biotin is severed, so that the conjugate containing the fluorescent complex portion breaks free into the solution.

The aforementioned liquid phase fluorescence measurement advantage permits a wider range of types of solid phases and materials to be selected because the